GTP Cyclohydrolase Feedback Regulatory Protein Controls Cofactor 6-Tetrahydrobiopterin Synthesis in the Cytosol and in the Nucleus of Epidermal Keratinocytes and Melanocytes

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(6*R*)-L-Erythro 5,6,7,8 tetrahydrobiopterin ($6BH_4$) is crucial in the hydroxylation of L-phenylalanine-, L-tyrosine-, and L-tryptophan-regulating catecholamine and serotonin synthesis as well as tyrosinase in melanogenesis. The rate-limiting step of $6BH_4$ *de novo* synthesis is controlled by guanosine triphosphate (GTP) cyclohydrolase I (GTPCHI) and its feedback regulatory protein (GFRP), where binding of L-phenylalanine to GFRP increases enzyme activities, while $6BH_4$ exerts the opposite effect. Earlier it was demonstrated that the human epidermis holds the full capacity for autocrine $6BH_4$ *de novo* synthesis and recycling. However, besides the expression of epidermal mRNA for GFRP, the presence of a functioning GFRP feedback has never been shown. Therefore, it was tempting to investigate whether this important mechanism is present in epidermal cells. Our results identified indeed a functioning GFRP/GTPCHI axis in epidermal keratinocytes and melanocytes in the cytosol, adding the missing link for $6BH_4$ *de novo* synthesis which in turn controls cofactor supply for catecholamine and serotonin biosynthesis as well as melanogenesis in the human epidermis. Moreover, GFRP expression and GTPCHI activities have been found in the nucleus of both cell types. The significance of this result warrants further investigation.

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INTRODUCTION

Both epidermal melanocytes and keratinocytes have the full capacity to synthesize and recycle (6*R*)-L-erythro 5,6,7,8, tetrahydrobiopterin ($6BH_4$) (Schallreuter *et al.*, 1994). $6BH_4$ is a crucial cofactor for the three aromatic hydroxylases including phenylalanine hydroxylase (EC 1.14.16.1), tyrosine hydroxylase (EC 1.14.16.2), and tryptophan hydroxylase (EC 1.14.16.4). It also regulates the nitric oxide synthases (EC 1.14.13.39) as well as glyceryl ether monooxygenase (EC 1.14.16.5). In addition, this cofactor plays a major role in skin pigmentation since the redox balance of $6BH_4$ directly influences tyrosinase (EC 1.14.18.1) activities (Wood *et al.*, 1995). $6BH_4$ is synthesized *de novo* in a three-step reaction via guanosine triphosphate (GTP) cyclohydrolase I (EC 3.5.4.16, GTPCHI), followed by 6-pyruvoyl-tetrahydropterin synthase (EC 4.6.1.10) and finally sepiapterin reductase (EC1.1.1.153). After $6BH_4$ has been utilized as a cofactor, it produces pterin 4a-carbinolamine due to the molecular transfer of oxygen. This metabolite is then fully recycled in a two step enzymatic reaction via pterin 4a-carbinolamine dehydratase (EC 4.2.1.96), and dihydropteridine reductase (EC 1.6.99.7).

GTPCHI is the rate-limiting enzyme in the *de novo* synthesis of $6BH_4$ catalyzing the conversion of GTP to 7,8 dihydroneopterin triphosphate in a series of complex reactions, beginning with the opening of the imidazole ring of GTP and the removal of the purine carbon atom 8 of GTP giving rise to formate release and finally yielding 7,8 dihydroneopterin triphosphate (Nar *et al.*, 1995; Thöny *et al.*, 2000; Rebelo *et al.*, 2003).

The *de novo* synthesis of $6BH_4$ is tightly controlled by GTPCHI feedback regulatory protein (GFRP). Already in 1984 it had been recognized that GTPCHI was under feedback inhibition by $6BH_4$, although the underlying mechanism and control was at first not fully understood (Bellahsene *et al.*, 1984). Later it was shown that the presence of high $6BH_4$ levels downregulates GTPCHI activity via GFRP, while L-phenylalanine upregulates this enzyme (Harada *et al.*,

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Abbreviations: $6BH_4$, (6R)-L-Erythro 5,6,7,8 tetrahydrobiopterin; GFRP, GTPCHI feedback regulatory protein; GTP, guanosine triphosphate; GTPCHI, GTP cyclohydrolase I; PBS, phosphate-buffered saline; RT, room temperature; TRITC, tetramethyl rhodamine isothiocyanate

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1993). Hence, GFRP controls an elegant feedback mechanism in the *de novo* synthesis of $6BH_4$.

The presence of GFRP has been detected in many tissues including the liver and the brain (Harada *et al.*, 1993; Milstien *et al.*, 1996; Kapatos *et al.*, 1999; Park *et al.*, 2002). Although the presence and function of $6BH_4$ *de novo* synthesis/recycling was demonstrated in the human epidermis already in 1994, translation and function of GFRP in epidermal cells has never been shown (Schallreuter *et al.*, 1994, 1998). Therefore, we asked the question whether a functioning GFRP/GTPCHI axis is also present in the human epidermis in control of $6BH_4$ *de novo* synthesis. Our results demonstrate for the first time that this feedback regulatory protein is indeed expressed and functioning primarily in basal/undifferentiated keratinocytes as well as in melanocytes while this mechanism is downregulated upon differentiation. These results are in agreement with the earlier observation that $6BH_4$ *de novo* synthesis/recycling is much more predominant in the basal layer compared to suprabasal layers (Schallreuter *et al.*, 1994). In addition, we detected GFRP together with GTPCHI in the nucleus of both cell types and we were able to show GTPCHI activity in this organelle. The nature of this observation is still not fully understood at this time point. Clearly this observation warrants further investigation.

RESULTS

GFRP protein is expressed *in situ*

Protein expression of GFRP in the human epidermis has never been reported. Using 3 mm punch biopsies from healthy controls with skin phototype III (Fitzpatrick classification), we here demonstrate the presence of this regulatory protein within the epidermal compartment utilizing immunofluorescence labelling with a specific GFRP antibody (Kalivendi *et al.*, 2005). The protein is present throughout the human epidermis but it is more predominant in the basal layer (Figure 1a). Image analysis of the fluorescence yields a 3-fold increase in intensity in this layer compared to suprabasal layers (Figure 2a). In order to test whether this protein is also present within melanocytes, we used the melanosomal gp100 protein (NKI/beteb) (Figure 1b) and followed the overlay of both GFRP and NKI/beteb. The result demonstrates the presence of GFRP in some but not all melanocytes, suggesting subpopulations within this cell type under *in situ* conditions (Figure 1c).

GFRP is expressed in human epidermal melanocytes and keratinocytes in the cytosol as well as in the nucleus

Melanocytes express GFRP under *in vitro* conditions in the dendrites and in the nucleus (Figure 3a). Electron microscopy coupled with immuno-gold labelling further demonstrated the presence of GFRP in the nucleus with multiple gold particles bound in this organelle and also on the nuclear membrane (Figure 3e). Moreover, a positive granular co-localization with gp100 (NKI/beteb) suggests the presence of this regulatory protein within the melanosomes (Figure 3b and c).

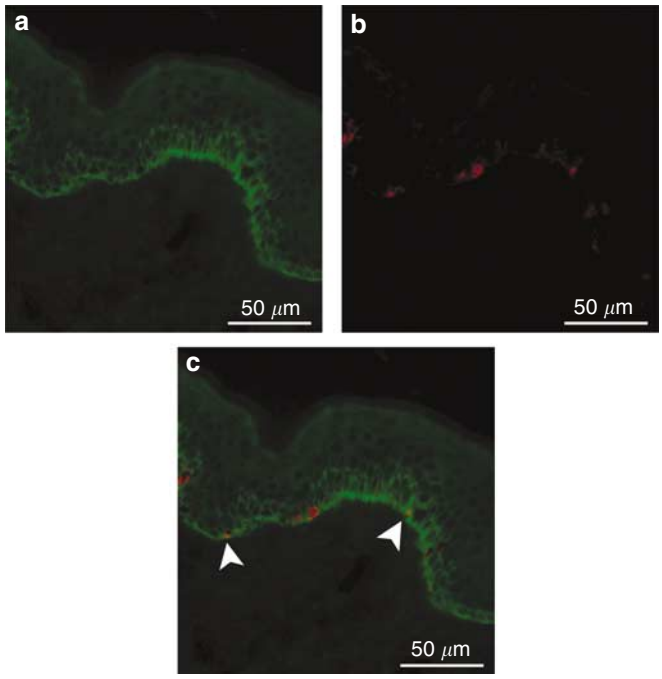

Figure 1. GFRP protein is expressed *in situ*. (**a**) GFRP (FITC labelling) is predominantly expressed in the basal layer of the epidermis with much weaker expression in the suprabasal layers, (**b**) NKI/beteb (TRITC labelling) shows melanocytes, and (**c**) overlay of (**a**/**b**) identifies colocalization (yellow) in some melanocytes (▲) suggesting the presence of a melanocyte subpopulation (original magnification × 400).

Keratinocytes express GFRP under *in vitro* conditions in the cytosol and in the nucleus in a diffuse and in a granular pattern. The expression is stronger in undifferentiated keratinocytes compared to differentiated cells (Figure 4a and d). This result is in agreement with the *in situ* observation (Figure 1a).

GFRP colocalizes with GTPCHI *in situ* and *in vitro*

Since it has already been demonstrated earlier that GTPCHI is transcribed and translated into a functioning protein in the human epidermis (Schallreuter *et al.*, 1994), we wanted to know whether the enzyme colocalizes with GFRP expression. Therefore, we utilized a specific GTPCHI antibody (Hesslinger *et al.*, 1998) in addition to GFRP labelling. The result shows expression of GTPCHI as expected throughout the human epidermis with a more pronounced expression in the basal layer (Figure 2b). This finding is in agreement with highest GTPCHI enzyme activities in undifferentiated keratinocytes as previously reported (Schallreuter *et al.*, 1994). Even more exciting was the overlay of both immunoreactivities which shows strong GTPCHI colocalization with its regulatory protein GFRP in the basal layer which is much weaker in suprabasal layers (Figure 2c). Moreover, both proteins colocalize in epidermal keratinocytes and melanocytes under *in vitro* conditions supporting the presence of GFRP feedback regulation for $6BH_4$ *de novo* synthesis in the epidermal compartment (Figures 4 and 5).

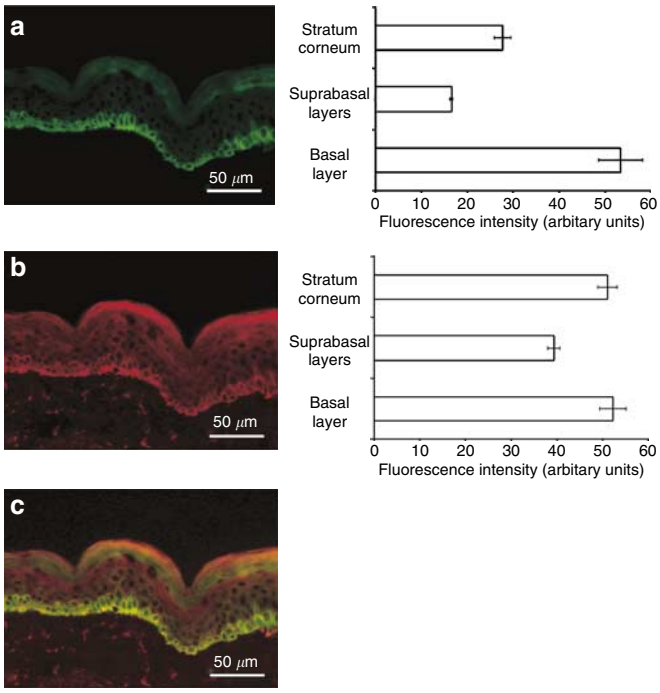

Figure 2. Epidermal GFRP and GTPCHI are co-expressed *in situ*. (**a**) GFRP (FITC labelling) expression with corresponding image analysis of the *in situ* immuno-reactivity, (**b**) GTPCHI (TRITC labelling) expression with corresponding image analysis of the *in situ* immuno-reactivity, and (**c**) overlay of (**a**/**b**) shows strongest colocalization of both proteins in the basal layer and in the stratum corneum. There is no colocalization in the suprabasal layers (original magnification ×400). (Units are expressed as arbitary units.)

Western blot analysis supports GFRP protein expression within the cytosol and the nucleus of epidermal melanocytes and keratinocytes

In order to substantiate the presence of GFRP in epidermal cells, Western blot analysis was employed. The protein was detected in cytosolic cell extracts as well as in nuclear extracts from melanocytes and keratinocytes. The bands at the 10 kDa molecular weight marker corresponded to the monomer of GFRP of 9.5 kDa (Figure 6) (Milstien *et al.*, 1996; Yoneyama *et al.*, 1997). This result was in agreement with the band found in human liver lysate (Abcam Ltd, Cambridge, UK) which was used as positive control (Figure 6 inset). Unfortunately, we could not further substantiate this result by specific blocking because no blocking peptide is currently available.

Epidermal GTPCHI enzyme activity is coupled to GFRP in human epidermal melanocytes and keratinocytes

In order to prove the functionality of the GFRP protein in melanocytes and keratinocytes, we followed neopterin formation by adding rhGTPCHI (Ichinose *et al.*, 1995) in the presence and absence of $6BH_4$ and L-phenylalanine. The rationale for this approach was that this co-factor is a negative regulator of GTPCHI, whereas L-phenylalanine is a positive upregulator (Harada *et al.*, 1993). The first step was to ensure that the cell extracts of both epidermal cells had indeed active GTPCHI activity. For this purpose we followed neopterin formation as outlined in the method section (Schallreuter *et al.*, 1994; Werner *et al.*, 1997). Both cell types showed GTPCHI activity (data not shown). Based on this result we then determined neopterin formation in the presence and absence of $6BH_4$ and L-phenylalanine by adding rhGTPCHI to test GFRP/GTPCHI coupling. The result showed indeed a significant decrease in neopterin formation with $6BH_4$ (Figure 7). Moreover, a significant increase was observed in the presence of L-phenylalanine in a concentration-dependent manner (Figure 7). Both effects were absent when $6BH_4$ and L-phenylalanine were not added (Figure 7). Based on these result we can conclude that epidermal keratinocytes and melanocytes have the capacity to control GTPCHI via GFRP which in turn regulates $6BH_4$ *de novo* synthesis and consequently catecholamine and serotonin synthesis as well as nitric oxide synthesis in these cells (Klatt *et al.*, 1992; Schallreuter *et al.*, 1992; Shimizu *et al.*, 1998; Slominski *et al.*, 2002, 2003; Gillbro *et al.*, 2004).

Nuclear extracts of epidermal melanocytes and keratinocytes have GTPCHI activity

After demonstration of GFRP and GTPCHI immuno-reactivity in the nucleus of epidermal keratinocytes and melanocytes by labelling and Western blot, it was tempting to test possible GTPCHI activity in this organelle. For this purpose, we followed again neopterin formation in nuclear extracts in the presence and absence of GTP, the natural substrate for GTPCHI. HPLC analysis showed a significant increase of the neopterin peak in the presence of GTP confirming GTPCHI activity in the nucleus of these cells (Figure 8). The significance of nuclear GTPCHI activities is currently not fully understood. Whether GFRP is indeed coupled to GTPCHI in this organelle is currently under further investigation.

DISCUSSION

Both epidermal melanocytes and keratinocytes hold the full capacity to synthesize and recycle $6BH_4$ (Schallreuter *et al.*, 1994). GTPCHI is the rate-limiting enzyme in the *de novo* synthesis of this cofactor catalyzing the conversion of GTP to 7,8 dihydroneopterin triphosphate. This enzyme is regulated at the transcriptional, translational, and also at the level of enzyme activity via GFRP (Ziegler *et al.*, 1990, 1993; Werner *et al.*, 1991; Togari *et al.*, 1992; Harada *et al.*, 1993; Gütlich *et al.*, 1994; Thöny *et al.*, 2000; Hwu *et al.*, 2003). Depending on the balance of L-phenylalanine and $6BH_4$, this protein can either up- or downregulate GTPCHI activity (Harada *et al.*, 1993; Yoneyama *et al.*, 1997; Yoneyama and Hatakeyama, 2001; Maita *et al.*, 2002, 2004). Hence, this mechanism leads to an intricate regulation of $6BH_4$ *de novo* synthesis.

The presence of a functioning GFRP feedback regulation has been documented in various cell types including liver and neuronal cells (Milstien *et al.*, 1996; Kapatos *et al.*, 1999; Park *et al.*, 2002), but except mRNA expression in epidermal melanocytes and keratinocytes (Schallreuter *et al.*, 1998) a functioning GFRP/GTPCHI axis has not been shown in these cells. From the data presented herein, we now show the

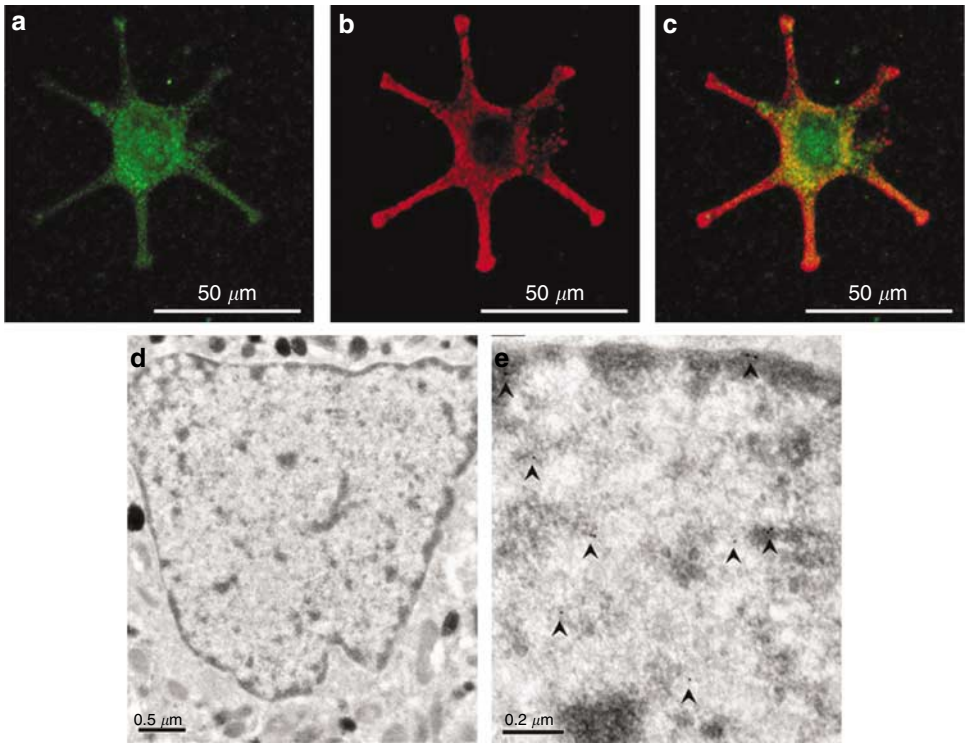

Figure 3. *In vitro* expression of GFRP in human epidermal melanocytes. (**a**) GFRP (FITC labelling) is expressed in a granular pattern throughout the entire cell including the nucleus, (**b**) NKI/beteb (TRITC labelling) identifies melanocytes, (**c**) overlay of (**a**/**b**) shows colocalization with NKI/beteb (yellow) suggesting the presence of GFRP in melanosomes (original magnification $\times 400$), (**d**) electron microscopy showing the nucleus of an epidermal melanocyte (bar = 0.5 μm), and (**e**) further magnification of nuclear region illustrating the binding of multiple gold particles within this region indicating GFRP presence within this organelle (bar = 0.2 μm).

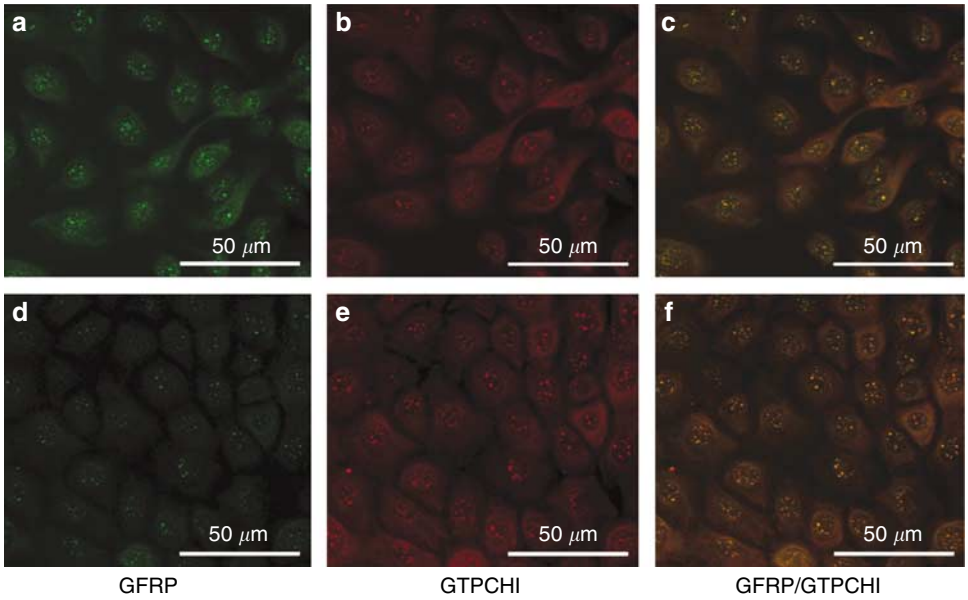

Figure 4. GFRP and GTPCHI expression in undifferentiated and differentiated keratinocytes. *Undifferentiated keratinocytes* (**a**) GFRP (FITC labelling), (**b**) GTPCHI (TRITC labelling), and (**c**) overlay of (**a**/**b**). The high expression of GFRP and GTPCHI in undifferentiated cells is in agreement with high GTPCHI activities as shown earlier (Schallreuter *et al.*, 1994). *Differentiated keratinocytes* (**d**) GFRP (FITC labelling), the expression is reduced upon differentiation in the cytosol as well as in the nucleus (**e**) GTPCHI (TRITC labelling), the expression does not differ in the cytosol and in the nucleus. (**f**) Overlay of (**d**/**e**).

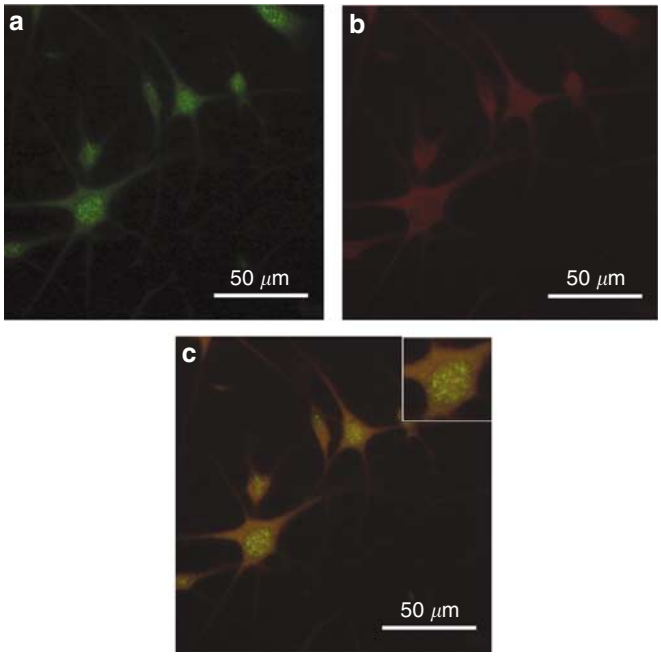

Figure 5. Co-expression of GFRP and GTPCHI in human epidermal melanocytes. (**a**) GFRP (FITC labelling), (**b**) GTPCHI (TRITC labelling), both proteins are expressed in a granular pattern in the cytosol and in the nucleus. (**c**) Overlay of (**a**/**b**) shows the strongest colocalization (yellow) in the nucleus indicating the presence of both proteins in this organelle.

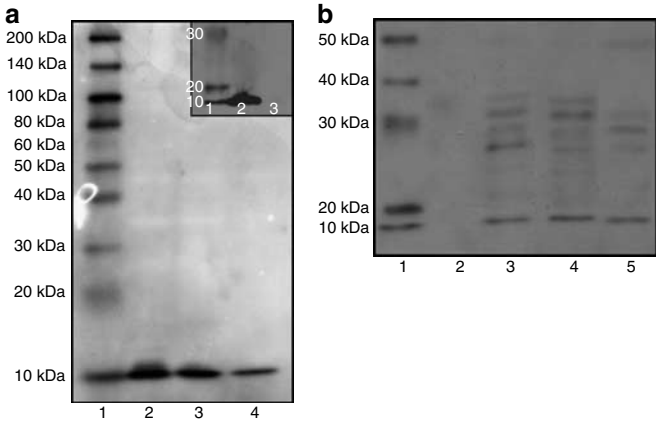

Figure 6. Western blot analysis confirms the presence of GFRP in the cytosol and in the nucleus of melanocytes and keratinocytes. (**a**) Analysis of cytosolic extracts. Lane 1 = ladder, lane 2 = undifferentiated keratinocytes, lane 3 = differentiated keratinocytes, and lane 4 = melanocytes. The inset shows the presence of GFRP in human liver lysate as a positive control. Lane 1 = ladder, lane 2 = positive control, and lane 3 = negative control. (**b**) Analysis of nuclear extracts. Lane 1 = ladder, lane 2 = negative control, lane 3 = undifferentiated keratinocytes, lane 4 = differentiated keratinocytes, and lane 5 = melanocytes. These results confirm the presence of GFRP in the cytosol and in the nuclei of epidermal cells. NB: Blocking peptides for GFRP are not available at this time.

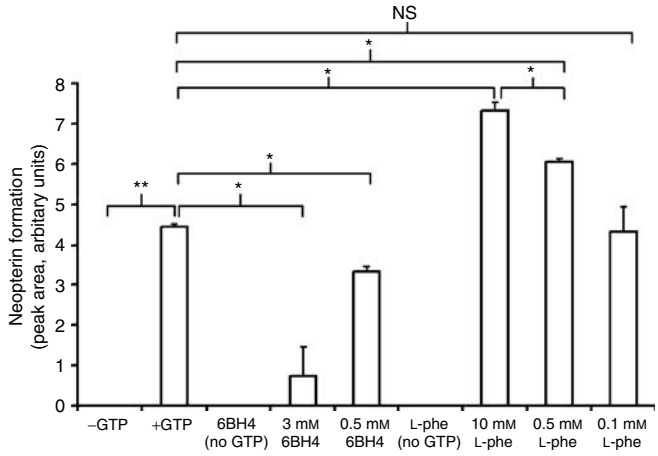

Figure 7. GFRP activity is present in human epidermal melanocytes. In the absence of GTP neopterin is not detected using HPLC analysis as outlined in methods. The addition of GTP leads to a significant increase in neopterin formation compared to the control which in turn mirrors GTPCHI activity (**$P<0.01$). Addition of $6BH_4$ the negative regulator of GTPCHI activity via GFRP results in a decrease in enzyme activity. GTPCHI is significantly upregulated in a concentration-dependent manner in the presence of L-phenylalanine (*$P<0.05$). This result proves positive GFRP feedback regulation on GTPCHI activity in melanocytes.

expression of this protein throughout the human epidermis with stronger localization of GFRP in the basal layer compared to suprabasal layers (Figures 1a and 2a). This result is in agreement with GTPCHI activities and with $6BH_4$ levels in the human epidermal compartment as reported previously (Schallreuter *et al.*, 1994). GFRP expression under *in vitro* conditions showed a very granular staining pattern in the cytosol as well as in the nucleus of both cell types. This result suggests that GFRP may be localized within vesicles (Figures 3a, 4a, and d). Colocalization with gp100 (NKI/beteb) indicated the presence of GFRP in melanosomes (Figure 3c). This result was supported by immuno-gold electron microscopy where gold particles were indeed found in these organelles (data not shown). However, the presence of GFRP in melanosomes needs further investigation.

In our attempt to test whether the immuno-reactive GFRP protein was actually coupled to GTPCHI enzyme activity, we used both regulators of this protein, that is $6BH_4$ (negative feedback) and L-phenylalanine (positive feedback) in cell extracts of keratinocytes and melanocytes. Our results proved indeed a significant reduction in the presence of $6BH_4$ while L-phenylalanine upregulated neopterin formation in a concentration-dependent manner (Figure 7). Therefore, we can conclude that GFRP/GTPCHI-positive feedback regulation is taking place in these cells (Figure 7). Clearly the expression of GFRP follows GTPCHI activities with higher enzyme activities in undifferentiated keratinocytes and in melanocytes underlining once more the importance of $6BH_4$ for the turnover of the essential amino acid L-phenylalanine to L-tyrosine for catecholamine and serotonin synthesis in cell proliferation and pigmentation (Schallreuter *et al.*, 1992, 1995; Slominski *et al.*, 2002, 2003; Gillbro *et al.*, 2004).

The presence of GFRP expression in the nucleus as shown by immuno-reactivity, electron microscopy, and Western blot is novel, but the function needs to be shown. In this context it is noteworthy that the presence of all enzymes for $6BH_4$

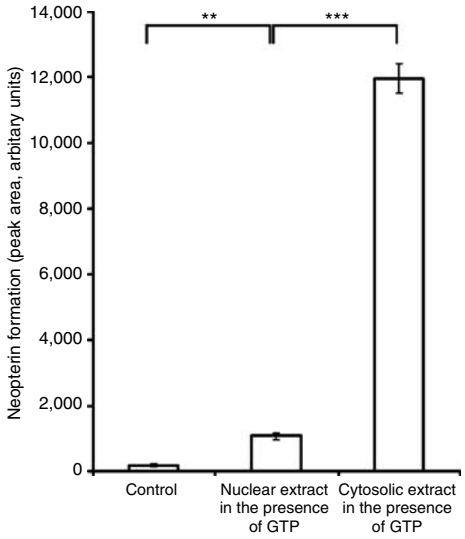

Figure 8. GTPCHI is active in the nucleus of human epidermal melanocytes. Nuclear extracts obtained from human epidermal melanocytes showed significant GTPCHI activity after addition of the substrate GTP, which was absent in the control extract (**$P<0.01$). Comparison of enzyme activities from nuclear and cytosolic extracts shows an 11-fold higher activity in the cytosol (***$P<0.005$). This result is based on two different extracts which were analyzed in duplicates.

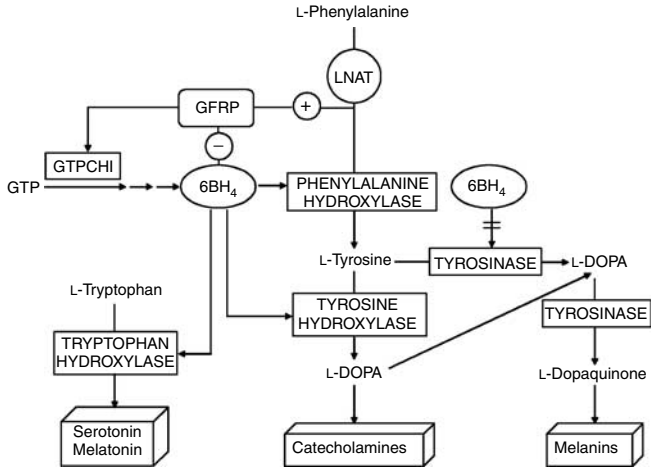

Figure 9. The importance of the GFRP/GTPCHI axis for catecholamine/serotonin synthesis and melanogenesis in human epidermal keratinocytes and melanocytes. L-phenylalanine enters the cell via the large neutral amino acid transporter (LNAT). Binding of L-phenylalanine to GFRP regulates $6BH_4$ *de novo* synthesis by increasing GTPCHI activity, whereas $6BH_4$ binding exhibits the opposite effect (Harada *et al.*, 1993; Yoneyama and Hatakeyama, 1998, 2001; Maita *et al.*, 2002, 2004). The cofactor is required for L-phenylalanine turnover to L-tyrosine via phenylalanine hydroxylase and for tyrosine hydroxylase isoform I to initiate catecholamine synthesis in keratinocytes and melanocytes as well as melanogenesis in melanocytes (Schallreuter and Wood, 1999; Marles *et al.*, 2003; Gillbro *et al.*, 2004). Moreover, $6BH_4$ is needed for the turnover of L-tryptophan to 5-OH-tryptophan finally yielding serotonin and melanotonin (Slominski *et al.*, 2002, 2003).

de novo synthesis was demonstrated in the nucleus of transfected COS-1 cells while only GTPCHI and 6-pyruvoyl-tetrahydropterin synthase were found in rat brain nucleus (Elzaouk *et al.*, 2004). These authors did not show GFRP in the nucleus. Owing to low amounts of nuclear extracts at this point, we were not able to test yet the coupling of GFRP/GTPCHI as shown in the cytosol. Further research is on the way in our laboratory to get a more detailed understanding for the presence of $6BH_4$ *de novo* synthesis and its role in this organelle.

In summary, human epidermal melanocytes and keratinocytes have the capacity to regulate $6BH_4$ *de novo* synthesis via the GFRP/GTPCHI axis. The expression of GFRP follows GTPCHI. This result also underlines the importance of intracellular L-phenylalanine uptake and turnover in these cells in order to provide sufficient L-tyrosine for both catecholamine synthesis and melanogenesis (Schallreuter and Wood, 1999; Marles *et al.*, 2003; Gillbro *et al.*, 2004). The importance of the GFRP/GTPCHI axis for $6BH_4$ *de novo* synthesis is summarized in Figure 9.

MATERIALS AND METHODS

Cell cultures

Primary cell cultures of human epidermal melanocytes and keratinocytes were established from full thickness skin received as surgical waste from routine cosmetic enhancements such as face-lifts and breast reduction tissue. Briefly, after removal of fat, the skin was washed in a solution containing 5% penicillin/streptomycin and 5% fungisone followed by dispase treatment (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, IN), for 12 hours at 4°C. The epidermis was peeled off and placed into sterile phosphate-buffered saline (PBS) and then transferred for trypsinization (1 × trypsin/EDTA, Sigma, Dorset, UK) for 10 minutes at 37°C. The cell suspension was centrifuged at $1,000 \times g$ and re-suspended in MCDB154 medium (Cascade Biologics, Mansfield, Nottinghamshire, UK) containing 1% penicillin/streptomycin and 0.1 mM calcium and finally seeded into T_{75} flasks (Scientific Laboratory Supplies, Nottingham, UK).

In order to establish pure keratinocyte or melanocyte cultures, cells were grown to 70–80% confluence, followed by sequential trypsination in MCBD154 medium containing either melanocyte or keratinocyte supplements (Cascade Biolobics, Mansfield, Nottinghamshire, UK). To promote differentiation of keratinocytes, the calcium concentration was increased to 2.5 mM and growth factors were removed (Pittelkow and Shipley, 1989). Differentiation was confirmed by the expression of the corresponding cytokeratins (Abcam, Cambridge, UK) (data not shown). For immuno-reactivity studies, keratinocytes or melanocytes were seeded into chamber slides (Nalge Nunc International, Naperville, IL) and cultured for 48 hours, followed by removal of the medium and a 3 × wash in sterile PBS, pH 7.4. Cells were fixed in ice-cold methanol for 5 minutes prior to the staining procedure.

Human skin biopsies

After signed consent, full thickness skin 3 mm punch biopsies were taken under local anesthesia from sun-unexposed skin of the inner proximal arm from healthy human volunteers ($n=3$; skin phototype III, Fitzpatrick classification) and embedded in optimal cutting temperature compound (Sakura, RA Lamb, Eastbourne, UK). The tissue was cut in 4–5 μm sections using a Leica CM3050 S cryostat. Sections were placed onto poly-L-lysine-coated slides and stored at −80°C until further use.

This study was approved by the local Ethics Committees and was in agreement with the Declaration of Helsinki Principles.

In situ double immunofluorescence studies

Frozen slides were air dried for 60 minutes at room temperature (RT), fixed prior to staining in ice-cold methanol and blocked in 10% normal donkey serum (NDS, Jackson Immuno Research Laboratories, Cambridgeshire, UK) for 90 minutes at RT followed by a 5 minutes wash in PBS. To demonstrate the presence of GFRP *in situ*, we used a well-characterized primary rabbit anti-human antibody (a kind gift from K. Hatakeyama, Japan) diluted 1:1,000 in 1% normal donkey serum and incubated for 90 minutes at RT. For GTPCHI *in situ* detection, we utilized a mAb rat anti-mouse antibody (a kind gift from C. Hesslinger, Germany) diluted 1:20 and incubated for 5 hours at RT. The slides were washed in PBS twice for 5 minutes followed by a wash in Tween 20 (Bio-Rad Laboratories, Life Science Group, CA) for 5 minutes with a final wash in PBS. Sections were air dried followed by incubation at RT for 1 hour with a fluorescent secondary antibody (Jackson Immuno Research Laboratories, Cambridgeshire, UK) in a dilution of 1:100 FITC-conjugated donkey anti-rabbit for GFRP or tetramethyl rhodamine isothiocyanate-conjugated donkey anti-rat (TRITC) for GTPCHI, followed by 3 × washing in PBS. In addition, we used for the detection of melanocytes the melanocyte-specific gp100 protein (NKI beteb, Monosan Antibodies, Buckinghamshire, UK) in a dilution 1:20 overnight at 4°C. Finally, the slides were dried and mounted in Vectashield Mounting Medium with 4,6-diamino-2-phenylindole (Vector Laboratories, CA) and viewed under a Leica DMIRB/E fluorescence microscope (Wetzlar, Germany). Images were captured using a color video camera coupled to a computer using Image Grabber PCI graphics program (Optivision, Osset, West Yorkshire, UK). In addition, some pictures were captured using a Nikon Eclipse 80i microscope with a DS-U101 Nikon camera coupled to a Nikon ACT-2U capture program (Nikon, Europe).

Paint Shop Pro™ 7 was utilized to merge the two different fluorochromes in order to follow possible colocalization.

In vitro immunofluorescence labelling of keratinocytes and melanocytes

For immuno-labelling of cultured cells the same procedure was used as described above. Keratinocytes were incubated for 2.5 hours at RT with anti-GFRP antibody at the dilution 1:5,000. Melanocytes were incubated overnight at 4°C with a dilution 1:500.

GTPCHI was used in the dilution 1:10 and both keratinocytes and melanocytes were incubated overnight at 4°C. In order to follow possible expression in melanosomes, melanocytes were incubated overnight at 4°C with the melanocyte-specific gp100 protein (NKI beteb) in the dilution 1:20.

Quantification of fluorescence intensity

For quantification of fluorescence intensity, we utilized IPLab v3.6.5a (Nikon, Europe) software following the manufacturer's protocol. The fluorescence was expressed in arbitary units.

Electron microscopy of GFRP utilizing immuno-gold labelling

Epidermal melanocyte cultures (passages 3–5) ($n = 2$) were harvested via trypsinization, followed by gentle centrifugation at 1,000 r.p.m. at 4°C. The pelleted cells were subsequently fixed for 1 hour at RT in a solution of 0.5% glutaraldehyde (Agar Scientific, Stanstead, UK) and 2% paraformaldehyde (Sigma, Dorset, UK) in 0.1 M sodium cacodylate buffer (Sigma, Dorset, UK), containing 0.027 mM $CaCl_2$ (Sigma, Dorset, UK), buffered to pH 7.4.

After fixation, cells were washed in PBS over 25 minutes, and rinsed in 0.1 M glycine (Sigma, Dorset, UK) in PBS, for 5 minutes, before being pelleted through 1% low melting point agarose (Bio-Rad Laboratories, CA) in PBS. The cell/agar blocks were dehydrated in graded series of ethanol and infiltrated with hydrophilic Unicryl resin (British BioCell International, Cardiff, Wales, UK), followed by polymerization using UV light (360 nm) of 2 × 8 W for 3 days at 4°C.

Ultrathin sections (90 nm) were cut using a Reichert-Jung ultramicrotome (Vienna, Austria) and mounted on 200 mesh nickel grids coated with a carbon film (Agar Scientific).

Sections processed as above were blocked in 10% normal goat serum and 2% BSA in PBS (pH 8.2) for 1 hour (1% normal goat serum in the same blocking buffer, was used to dilute the primary and secondary antibodies). The sections were washed twice in PBS containing 2% BSA (pH 8.2) and incubated with the GFRP primary antibody at 1:200 for 18 hours at 4°C. Following incubation, the sections were washed in 2% BSA in PBS, and incubated in a 1:60 dilution of the secondary goat anti-rabbit antibody conjugated to 10 nm gold particles (British BioCell International, Cardiff, Wales, UK) for 1 hour at RT. Following a final wash in distilled water, the sections were lightly counterstained with 2% uranyl acetate and Reynold's lead citrate solution and examined and photographed using a JEM-1200 EX transmission electron microscope (Jeol Tokyo, Japan).

Whole cell and nuclear extract preparation from melanocyte and keratinocyte cell cultures

Medium was aspirated from the flask and washed with ice-cold PBS containing phosphatase inhibitors. Cells were gently detached and centrifuged at 1,000 × *g* for 10 minutes. The pellet was resuspended in reaction buffer (50 mM Tris-HCl pH 7.8, 300 mM KCl, 2.5 mM EDTA, and 10% glycerol) and homogenized on ice. The homogenate was then centrifuged for 1 hour at 76,000 × *g* and the supernatant obtained was used as the whole-cell extract which was aliquoted and stored at −80°C until further use.

Nuclear extracts from undifferentiated keratinocytes and melanocytes were prepared using a nuclear extraction kit (Active Motif, Rixensart, Belgium) following the manufacturer's instructions. The cell pellet was obtained as described above and was resuspended in hypotonic buffer (Active Motif, Rixensart, Belgium) and incubated for 15 minutes on ice, followed by thorough mixing and finally centrifuged at 22,000 × *g* for 30 seconds. The pellet was resuspended in complete lysis buffer for 30 minutes on ice and then vortexed and centrifuged at 13,000 × *g* for 10 minutes. The supernatant collected was used as the nuclear extract. In order to test the purity of the extract, the lactic dehydrogenase assay was utilized (Howell *et al.*, 1979). This enzyme is only active in the cytosol and therefore a good marker for detection of cytosolic contamination. The protein content was determined at the optical density 280 nm using the method of Kalb and Bernlohr (1977).

Western blotting

Cytosolic and nuclear extracts from keratinocytes and melanocytes were separated in 10% SDS-PAGE and proteins transferred

to a polyvinylidene difluoride membrane (Immobilon™-P Millipore, Bedford, UK). The membrane was blocked with 0.5% gelatine in Tris-buffered saline containing 0.1% (v/v) Tween-20 buffer (150 mM NaCl, 20 mM Tris, 0.047% Tween, pH 7.4) and incubated overnight with rabbit anti-human GFRP antibody (dilution 1:20,000). The blot was washed and incubated for 1 hour at RT with an anti-rabbit IgG peroxidase-conjugated antibody (Fc specific, dilution 1:5,000, Cell Signalling Technology Inc., Beverley, MA). Visualization of GFRP bands was performed using modified enhanced chemiluminescence fixed on a film sheet (X-OMAT™ Kodak, USA).

Neopterin analysis in whole cell extract from keratinocytes and melanocytes to follow coupling of GFRP/GTPCHI

The method used was adapted from Werner *et al.* (1997) and slightly modified. For this assay cell extract from melanocytes and keratinocytes was utilized. The reaction contained 110 μl of cell extract, 1 μl recombinant human GTPCHI (a generous gift from Dr Ichinose, Japan) (Suzuki *et al.*, 1999), 300 μM GTP (Sigma, Aldrich, UK) in a total volume of 120 μl. In addition, differing concentrations of $6BH_4$ (3 and 0.5 mM final concentration) and L-phenylalanine (10, 0.5, and 0.1 mM final concentration) were added to see negative and positive regulation via GFRP. The reaction was incubated for 2.5 at 37°C in the dark. The pH was adjusted to 3 with 1 N phosphoric acid followed by oxidation with 1% iodine and 2% potassium iodide at RT in the dark. The oxidation was stopped with 3% ascorbic acid and the sample was dephosphorylated using alkaline phosphatase (Roche Diagnostics GmbH, Penzberg, Germany). Prior to HPLC analysis the samples were de-proteinized and finally analyzed using a Dynamax HPLC system and fluorescence detector (Hitachi RF-535, Shimadzu, Milton Klynes, Bucks, UK) along with sphereclone columns (Phenomenex, Macclesfield, Cheshire UK). The mobile phase contained 3% methanol, 0.1% phosphoric acid (1 M) in HPLC grade water and the organic solvent was HPLC grade methanol (Fisher Scientific, Loughborough, UK). The detector was set at an $\lambda_{ex_{345}}$ and $\lambda_{ex_{450}}$. Determination of enzyme activity was based on two independent assays.

GTPCHI activity in nuclear extracts from keratinocytes and melanocytes utilizing HPLC

The method used was as described above, however no rhGTPCHI was added and GTP was added in excess (2 mM final concentration). Neopterin detection was carried out under the same HPLC conditions as described above.

Statistical analysis

Data from multiple experiments are expressed as means $\pm$ SE Statistical significance was determined with paired and unpaired Student's *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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